COBISS Code 1.01

DOI: 10.14720/aas.2014.103.2.9
Agrovoc descriptors: plant production,wheats,triticum,variaties,mycotoxins,toxic substances,biological contamination,contaminationb wheat flour,flours,cropping systems,crop management

Agris category code: q03,h20

The impact of wheat production on the occurrence of mycotoxins DON (deoxynivalenol) and ZEA (zearalenone) on wheat grains (*Triticum aestivum* L.)

Lena TAJNŠEK[1], Marjan SIMČIČ[2], Anton TAJNŠEK[3]

Received June 06, 2014; accepted July 21, 2014.
Delo je prispelo 06. junija 2014, sprejeto 21. julija 2014.

ABSTRACT

The current study has been conceived to draw attention to the factors that should be avoided in the production of cereal grains (such as high doses of nitrogen) by analysing differences in cereal grain contamination with mycotoxins DON (deoxynivalenol) and ZEA (zearalenone), depending on methods of wheat production. We studied the occurrence of DON and ZEA at very different production intensities in the 'Reska' and 'Savinja' wheat cultivars which were grown in a long-term field experiment (designed in 1992) at Jable near Ljubljana in the years 2006 and 2008. The cultivars 'Reska' and 'Savinja' have been considered in crop rotation of wheat-maize-oats in 5 methods of production with variants ranging organic up to intensely conventional. The results obtained have shown that adequately chosen agri-technical measures significantly reduced the risk of contamination of wheat flour by the mycotoxins DON and ZEA. Too abundant N (nitrogen) fertilization of either inorganic or organic origin is to be avoided. The extensive wheat growing reduces considerably the risk of DON and ZEA occurrence in comparison to the intensive one. Cultivar plays an important role in this process. The comparison of both cultivars has shown that the cultivar 'Savinja' was more resistant to the contamination with DON and ZEA than the cultivar 'Reska'.

Key words: mycotoxins, deoxynivalenol, zearalenone, wheat, nitrogen fertilization, food safety, production method

IZVLEČEK

VPLIV OKOLJSKIH DEJAVNIKOV NA POJAVNOST MIKOTOKSINOV DON (DEOKSINIVALENOL) IN ZEA (ZEARALENON) NA ZRNJU PŠENICE *(Triticum aestivum* L.*)*

V raziskavi smo analizirali razlike v kontaminiranosti žitnih zrn z mikotoksinoma DON (deoksinivalenol) in ZEA (zearalenon) v odvisnosti od načina pridelovanja, da bi opozorili na okoljske dejavnike, kot na primer visoke odmerke dušika, ki bi se jim morali v pridelavi pšenice izogibati. Pojavljanje mikotoksinov DON in ZEA smo proučevali v letih 2006 in 2008 pri različnih postopkih pridelovanja pšeničnih kultivarjev Reska in Savinja v okviru trajnega poljskega poskusa, zasnovanega leta 1992 v Jablah pri Ljubljani. Kultivarja Reska in Savinja sta bila posejana v kolobarju pšenica-koruza-oves v 5-ih postopkih pridelovanja, ki vključujejo variante, od ekološko prijaznih do intenzivnih konvencionalnih. Rezultati so pokazali, da lahko z ustreznimi agrotehničnimi ukrepi pomembno zmanjšamo tveganje za pojav kontaminacije pšenične moke z mikotoksinoma DON in ZEA. Pri ekstenzivni pridelavi pšenice je tveganje za pojav DON in ZEA občutno manjše kot pri intenzivni. Pomembno vlogo ima tudi kultivar: primerjava kultivarjev je pokazala, da je Savinja bolj odporna na kontaminacijo z DON in ZEA kot Reska.

Ključne besede: mikotoksini, deoksinivalenol, zearalenon, pšenica, varnost hrane, gnojenje z dušikom, metode pridelovanja

[1] Bodešče 10, SI-4260 Bled, Slovenia, e-mail: lena.tajnsek@gmail.com; General Hospital Jesenice, Cesta maršala Tita 112, SI - 4270 Jesenice, Slovenia, e-mail: lena.tajnsek@sb-je.si

[2] Univ. of Ljubljana, Biotechnical Fac., Dept. of Food Science, Jamnikarjeva 101, SI-1111 Ljubljana, Slovenia, e-mail: marjan.simcic@bf.uni-lj.si

[3] Murgle 26, SI-1001 Ljubljana, Slovenia, e-mail: tone.tajnsek@bf.uni-lj.si

The article is part of doctoral dissertation of postgraduate study of Biological and Biotechnical Sciences, Field Food Science and Technology, Biotechnical Faculty, University of Ljubljana, supervisor: prof. dr. Marjan Simčič.
Prispevek je del doktorskega dela podiplomskega študija bioloških in biotehniških znanosti, področje živilstva, Biotehniška fakulteta, Univerza v Ljubljani. Mentor: prof. dr. Marjan Simčič.

1 INTRODUCTION

When eating food, humans and animals are exposed to various mycotoxins which are formed as degradation products of the metabolism of the *Fusarium* spp. present in cereals. Long-term intake of cereal products (bread, pasta, biscuits, etc.) that are contaminated with these mycotoxins may be the cause of serious developmental and hormonal disorders, chronic poisoning, malignant tumours and other diseases, as well as deformities (Smith et al., 1994; Gregorčič et al., 2009; Casteel and Rottinghaus, 2000). Williams and Hammitt (2001) considered the consumers to be insufficiently aware of the threats posed by the presence of mycotoxins in food. They stated that the consumers were certain that it were primarily the pesticides and not the presence of mycotoxins that put their health at risk, however, the authors' opinion was just the opposite: human health was exposed to increased risk due to the potential effects of mycotoxins rather than to the residues of fungicides in food.

Mycotoxins which are the result of the secondary metabolism of mycotoxicogenic mold (inter alia fungi *Fusarium* spp.) occur in cereals (often in wheat, maize, barley and triticale) as fungal infections. Visible signs of disease (FHB - *Fusarium* head blight - is a devastating disease of wheat with spikelets exhibit symptoms of premature bleaching shortly after infection by the fungal plant pathogen of genus *Fusarium* spp.*)* that may be present in all parts of the plant, especially in the grains and inflorescences (spikes, cobs and wiper), reduce the quantity and quality of crop yields. Among the most problematic mycotoxins belong mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) which are formed by the metabolism of *Fusarium* spp. (*F. graminearum* Swabe, *F. culmorum* (W.G. Smith) *Sacc.*, etc). DON poses a significant threat to the health of domestic animals and humans because of its deleterious effects on the digestive system and disturbances in normal cell function by inhibiting protein synthesis. DON has high cytotoxic and immunosuppressive properties. Humans consuming flour made from wheat contaminated with DON will often demonstrate symptoms of nausea, fever, headaches, and vomiting. At high doses, DON induces acute gastrointestinal toxicity; chronic, at low doses immunotoxicity has been reported (Nogueira da Costa et al., 2011); at the molecular level DON disrupts normal cell function by inhibiting protein synthesis via binding to the ribosome and by activating critical cellular kinases involved in signal transduction related to proliferation, differentiation and apoptosis (Waśkiewicz et al., 2014). Because of concerns related to DON, the United States FDA has instituted advisory levels of 5 µg/g for grain products for most animal feeds and 10 µg/g for grain products for cattle feed (Waśkiewicz et al., 2014). ZEA is a phytohormone, which displays, apart from its anabolic properties, mainly estrogenic effects. Because of its estrogenic properties, ZEA may induce fertility disorders in animals with clinical signs of hyperestrogenism – an aspect of a disease which although reported mainly in hogs, is described in other species such as cow, horse and sheep, too. The potential health risk for man induced by this mycotoxin, which is taken up with foods of vegetable or animal origin, is extensively discussed. As an immunotoxic compound similar to estrogen and some endocrine disruptors ZEA has the toxic effects on the immune function, promote reduction in body weight gain (which is not fully explained by diminished food consumption), cause thymic atrophy with histological and thymocyte phenotype changes and decrease in the B cell percentage in the spleen and also weaken the antibody production and peroxide release by macrophages (Hueza et al., 2014). These two mycotoxins have therefore become the subject of worldwide intensive research in recent decades (Atroshi et al., 2002; European Commission, 1999; Smith et al., 1994; Srey at al., 2014).

A tolerable daily intake (TDI) for mycotoxins DON and ZEA has been evaluated by the Scientific Committee for Food (SCF); a TDI for DON of 1 µg/kg body weight and a provisional TDI of 0.2 µg/kg body weight for ZEA (Commission regulation, 2004).

Analysis of the levels of ZEA and DON in wheat and other cereals has become a legal obligation in many countries, including Slovenia. In 2006, the European Union decreed the establishment of the monitoring of contamination of cereals and cereal products as well as the maximum content of these

mycotoxins in food products. The maximum permissible content in wheat for adults is 750 µg DON kg^{-1} of flour and 75 µg ZEA kg^{-1} of flour. The criteria are stricter for children, with less than 200 µg DON kg^{-1} of flour and below 20 µg ZEA kg^{-1} of flour being allowable (Commission Regulation, 2006a).

Although scientists have conducted intensive research into ways in which to avoid the contamination of crops and mould-resistant plant species and new fungicides have been developed, no highly effective protective methods of avoiding the occurrence of mycotoxins in crops have been ascertained as yet (Jakovac-Strajn et al., 2004). Nevertheless suspensions or solutions with 1 % of Chinese galls (*Galla chinensis)* or 1 % of tannic acid had an antifungal toxicity and inhibited growth of *Fusarium graminearum* by 98–100 % or by 75–80 % in wheat that was artificially inoculated with *Fusarium graminearum* and *F. crookwellense* and then treated with 5 % suspensions of tannic acid and Chinese galls, whereas dried bark from buckthorn (*Rhamnus catharticus* L.) showed no effect at this concentration. In field experiments with two wheat varieties and artificial or semi-natural inoculations, mean DON reductions of 66 % (with tannic acid) and 58 % (with *Rhamnus catharticus* L.) were obtained (Forrer et al., 2014).

Previous studies have paid too little attention to the impact of production methods on the occurrence of mycotoxins in wheat grain; such occurrence has been discussed primarily in relation to the precipitation, to the crop rotation and to the cultivars and has rarely assessed the differentiated and simultaneously precise circumstances and methods of production. Rather than examining individual modes of production, surveys have been directed at the study of the impact of other factors, such as warm and humid weather, crop rotation of cereal grain crops or cultivars with differences in the resistance to mycotoxins (Schachermayr and Fried, 2000). Some scientists in Slovenia have investigated the influence of the environment on the occurrence of mycotoxins in wheat crops. Zemljič et al. (2008) found that location played an important role in the occurrence of DON while other mycotoxins, including ZEA, have not been identified in crop yields. Gregorčič et al. (2009) examined mycotoxin contamination in wheat and detected DON in the majority of the tested samples, ZEA in half of the samples and the threshold values of both DON and ZEA were exceeded in one fifth of all samples. Jakovac-Strajn et al. (2010) studied grain contamination with mold and the incidence of mycotoxins in cereals cultivated by farmers and used for animal feed. It was found that 73 % of the samples were contaminated, the majority with the mold *Fusarium* spp. causing the mycotoxin DON, and to a lesser extent ZEA and other toxins. Kalcher-Tavčar et al. (2007) examined the contamination of animal feed and, inter alia, found an average content of 178 µg ZEA kg^{-1} grains in 42 % of all tested samples.

The above findings confirm that the control of mycotoxins in grain production is necessary and justified in Slovenia. By following the principles of good agricultural practice and the optimization of production methods, the occurrence of *Fusarium* fungi can be reduced and the risk factors for the occurrence of mycotoxins can be limited to the lowest level (Commission Regulation, 2007).

This investigation forms part of a wider research into the relationship between the occurrence of DON and ZEA and the methods of production. Its purpose is the identification of environments and production systems in which mycotoxins occur with greater or lesser intensity. The aim of the study was to find one or more appropriate agricultural practices of wheat production in order to avoid the contamination with mycotoxins DON and ZEA promoting fungi.

2 MATERIALS AND METHODS

In order to conduct the agricultural labour research, we used the static long-term field experiment, which is a part of the network of international field trials known as ′Internationaler organischer Stickstoff-Düngungsversuch/International organic nitrogen fertilization long-term experiment′ (IOSDV), which have been conducted at Jable near Ljubljana (SI) since 1992. The design of this long-

term field experiment was described in detail in the previous descriptions together with their environmental parameters (Tajnšek, A., 2003; Tajnšek L. and A. Tajnšek, 2004; Tajnšek L., 2004).

The location of IOSDV at Jable is an experiment in the alpine climate area where long periods of drought rarely occur. Depending on the texture of the soil, the average annual rainfall is often too high (1343 mm a^{-1}, with an upward trend; Tajnšek et al., 2013) to ensure crops of wheat that are stable and of good quality, and the temperature is suitable (9.5 ºC a^{-1}). Given the long-term average rainfall and temperature, the climate conditions for the production of high-quality wheat at Jable, the fluctuations of precipitation between different years are higher than in those regions of the world where the most favourable conditions for the production of wheat exist.

The experiment has been conducted in the rotation of three crops (maize-wheat-oats) in three repetitions of basic parcels, each measuring 30 m^2. Wheat, maize and oats are sown each year in one of the three plots (fields, each measuring 1800 m^2) in crop rotation, so that various crops returns every third year to the same parcel. Each of these three plots is divided into three blocks and each of the blocks is divided into 10 basic parcels (of 30 m^2) with specific production methods. The process of production of each of these 10 basic parcels of all three field crops took place in a specific manner throughout the period of the experiment. From all these 10 methods of production 5 methods were chosen for the purposes of this study (Table 1). The specificity of permanent experiments conceived in this way is such that the methods of production lead to systematic differences between the level and the quality of crops in each of the years studied.

Table 1: Methods of production at IOSDV, Jable (chosen methods), 1993 – 2008

Preglednica 1: Metode pridelovanja v poskusu IOSDV Jable (izbrane metode), 1993 - 2008

The method of production	Code of production method	Annual rate of mineral nitrogen (N-min) for wheat	Level of production method with mineral nitrogen (N-min)
System A:			
Without organic fertilization	AN0	0	Without fertilization with N-min
System B:			
Fertilization with FM**			
(300 dt ha^{-1} FM every third year; before sowing maize)	*BN0**	0	Without fertilization with N-min
	BN2	130	Moderate rate of N-min
System C:			
Ploughed in maize stalk, straw of wheat, barley and oats; 60 kg ha^{-1} N-min before sowing oilseed radish	*CN0**	0	Without fertilization with N-min
	CN2	130	Moderate rate of N-min

In addition to all parcels in the fertilization experiment 100 kg P2O5 ha-1 a-1 and 180 kg K2O ha-1 a-1 were given
* Methods written in italics, BN0 and CN0, are the approximation of the principles of sustainable farming
**FM = farmyard manure

In Table 1, management without nitrogen and organic fertilization is indicated by code A, management with FM is indicated by code B and management with ploughing in the by-products (of wheat, barley or oats straw and of maize stalk) and oilseed radish as a green manure after harvesting barley or oats before planting maize, is indicated by code C. Wheat yield obtained in the years 2006 and 2008 was used to analyse the impact of production procedures on the occurrence of mycotoxins DON and ZEA. During these years, two cultivars of wheat, 'Reska' and 'Savinja', were sown in the long term experiment IOSDV; so both cultivars were included in our study which enables additional comparisons between the two cultivars.

Slovenian cultivar 'Reska' was acknowledged in 1996 (Grižon et al., 2011); characteristics of the cultivar are thick straw and thick grains, the absolute mass more than 44 g, a high protein content, up to 17.3 % and sedimentation value 25-32 ml (Tajnšek et al., 2010). Comparison with the standard at that time (cultivar 'Marija') showed that 'Reska' is of a very good quality for baking bread (Pavlic Nikolić, 2005). Because of long awns on the ear the cultivar 'Reska' is suitable especially for the areas where there is a greater risk of yield lowering caused by birds and wildlife. The cultivar is very resistant to high temperature stress even at very high temperature (Ristic et al., 2008).

Cultivar 'Savinja' is also a Slovenian cultivar, acknowledged in 2010 (Grižon et al., 2011). The cultivar is fertile in particular at the subalpine climate conditions with moderate rates of nitrogen (Čergan and Tajnšek, 2010). Depending on baking quality cultivar 'Savinja' is a typical improved cultivar and a good bread making cultivar (Tajnšek A. and Tajnšek L., 2011).

The years 2006 and 2007 at the location at IOSDV Jable were more dry during the period of flowering-ripening than year 2008 (average of April-July 2006: precipitation 98.5 mm, temperature 15.9°C; average of April-July 2007: precipitation 81.5 mm, temperature 16.7 °C; average of April-July 2008: precipitation 168.7 mm, temperature 15.9 °C) (SURS (Statistical Office of the Republic of Slovenia), 2011), thus for the analysis of wheat grains contamination with mycotoxins DON and ZEA the year 2007 (with the driest flowering-ripening period) was set aside, as several researchers found that wet weather had a significant impact on the contamination of wheat with mycotoxins DON and ZEA (Whitlow and Hagler 2009, Prandini et al. 2009). In both years studied, samples of wheat grains (1.5 - 2 kg) were taken from the harvested wheat from all plots after weighing, and stored in freezers at T = -20 °C prior to the analysis.

For the mycotoxicologic analysis of the presence of DON and ZEA a total of 60 grains samples (of 1.5 - 2 kg) were collected from basic parcels in both years. One half of the samples belonged to the cultivar 'Savinja' and the other half to the cultivar 'Reska'. Appropriate flour samples were prepared prior to laboratory analysis. Wheat grains were ground in the Brabender wheat mill suitable for grinding smaller size samples (MPI R. O. »Tehnićke usluge« Tip: S – 150 M, Atest: TU 78/1). The flour was separated by German typisation (DIN 10355) into white flour (type 405), dark flour (type 1050) and bran which was not sent for further analysis. From every wheat sample 110 – 120 g of white and dark flour were ground and weighed and a total of 120 flour samples were sent for the analysis of DON and ZEA mycotoxin content to the reference laboratory LUFA Speyer in Germany in which 240 chemical analysis were performed; i.e. 120 for DON and 120 for ZEA.

The standardized method used for detection of the mycotoxin DON in wheat flour developed in the reference laboratory LUFA Speyer is called ELISA LUFA SP 22005 (limit of detection and quantification is 200 ppm / 200 µg DON kg^{-1} flour) and that used for detection of the mycotoxin ZEA is called ELISA LUFA SP 22006 (limit of detection and quantification is 5 ppm / 5 µg ZEA kg^{-1} flour). The quantification of the mycotoxins content by those methods is based on competitive ELISA (the enzyme-linked immunosorbent assay), where a solid-phase enzyme immunoassay (EIA) is used to detect a presence of mycotoxins in a wet sample, based on the colour change reaction of the sample (Usleber et al., 1991; see also In Vitro Test, R-Biopharm AG, Darmstadt, and http://www.r-biopharm.com).

A competitive enzyme immunoassay used for the quantitative analysis of DON in wheat is RIDASCREEN®FAST DON (Art. No.: R5901, 96

wells / R5902, 48 wells) and the one used for the quantitative analysis of ZEA in wheat is RIDASCREEN® Zearalenon (Art. No.: R1401).

The RIDASCREEN®FAST DON can't differentiate between DON and 3-acetyl-DON (cross reactivity 213 %) and has a negligibly low or no cross reactivity to other related substances such as Nivalenol, 15-acetyl-DON or Triacetyl-DON.

The specificity of the RIDASCREEN® Zearalenon test was established by analysing the cross reactivity to corresponding mycotoxins (100 % for ZEA, 41.6 % for α-zearalenol, 27.7 % for zearalenol and 13,8 % for β-zearalenol).

All reagents for both enzyme immunoassay – including standards – are contained in the test kit in sufficient quantity for 96 determinations, including standards. A microtiter plate spectrophotometer is required for quantification.

Sample preparation include extraction and filtration (and dilution at sample preparation for determining ZEA), time requirement for sample preparation (for 10 samples) is 10 minutes for DON and 20 minutes for ZEA.

Test principle is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-DON antibodies and with specific antibodies to ZEA. DON/ZEA standards or sample solutions, DON/ZEA enzyme conjugate and anti-don antibodies are added. Free DON and DON-enzyme conjugate compete for the DON-antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-DON antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Substrate/chromogen is added to the wells, bound enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a colour change from blue to yellow. The measurement is made photometrically at 450 nm. The absorbance is inversely proportional to the DON concentration in the sample.

Materials required for determining DON and ZEA are reagent distilled or deionized water for determining DON and methanol (for determining ZEA) and equipment followed: microtitre plate spectrophotometer (450 nm), graduated cylinder-for DON (plastic or glass, 100 ml, 1 l), glassware for preparing sample extract: filter funnel and 50 ml flask for DON and 100 ml flask for ZEA, grinder (mill), Ultra-Turrax or equivalent shaker-optional for DON (shaker required for ZEA) filter paper (Whatman No. 1 filter) and variable 20-200 µl and 200-1000 µl micropipettes. Equipment required additionally for determining ZEA are rotary evaporator or another equipment for evaporation of solvents, pasteur pipettes and graduated pipettes are required.

Table 2: Reagent required for the determination of mycotoxins DON and ZEA in wheat competitive enzyme immunoassay RIDASCREEN®FAST DON and RIDASCREEN® Zearalenon used for the quantitative analysis of DON and ZEA in wheat

Preglednica 2: Potrebni reagent pri določanju mikotoksinov DON in ZEA v pšenici z encimsko imunskim testoma RIDASCREEN®FAST DON in RIDASCREEN® Zearalenon

Reagent*	For determining DON	For determining ZEA
Microtitre plate	96 or 48 wells (12, R5901 or 6 strips, R5902 resp., with 8 removable wells each)	96 wells coated with antibodies against ZEA (12 strips with 8 wells each)
Standard solutions	5 x DON standard solutions (1.3 ml each): 0 ppm -zero standard, 0.222 ppm, 0.666 ppm, 2 ppm, 6 ppm DON in water, ready to use; the dilution factor 20 for the sample has already been considered, therefore, the DON concentrations of samples can be read directly from the standard curve.	6 x standard solutions (1,3 m each): 0 ppt-zero standard, 50 ppt, 150 ppt, 450 ppt, 1350 ppt, 4050 ppt ZEA in aequeous solution
Conjugate	6 ml, R5901 and 3 ml, R5902; (red cap) peroxidase conjugated DON; ready to use	0.7 ml; (red cap); peroxidase conjugated ZEA concentrate
Anti-mycotoxin antibody	1 x anti-DON antibody (6 ml, R5901 and 3ml R5902; ready to use)	/
Substrate / Chromogen	Substrate / chromogen (10 ml; stained red, brown cap)	Substrate (7 ml; contains urea peroxide, green cap) Chromogen (7 ml) contains tetramethylbenzidine (blue cap)
Stop solution	14 ml; contains 1 N sulphuric acid; yellow cap	14 ml; contains 1 N sulphuric acid; yellow cap
Buffer	Buffer salt (washing buffer) for preparation of a 10 mM phosphate buffer (pH 7.4); Contains 0.05 % Tween 20.	Buffer 1 (50 ml) sample and conjugate dilution buffer (white cap)

*All the reagents from the Table 2 are contained in each kit (sufficient materials for as many as 91 or 43 analysis, plus 5 standard analysis; storage of kit at 2-8 ºC).

The preparation of samples is carried out according to the following steps in the Table 3 (the samples should be stored in a cool place, protected of light; a representative sample should be ground and thoroughly mixed prior to proceeding with the extraction procedure):

Table 3: Steps of determination of mycotoxins DON and ZEA in wheat competitive enzyme immunoassay RIDASCREEN®FAST DON and RIDASCREEN® Zearalenon used for the quantitative analysis of DON and ZEA in wheat

Preglednica 3: Postopki priprave vzorca za določanje mikotoksinov DON in ZEA v pšenici z encimsko imunskim testoma RIDASCREEN®FAST DON in RIDASCREEN® Zearalenon

Step	For determining DON	For determining ZEA
Weighing 5 g of ground sample into a suitable container	And addition of 100 ml of distilled water (sample size may be increased if required, but the volume of water must be adapted accordingly: e.g. 25 g in 500 ml of distilled water or 50 gin 1000 ml of distilled water)	And addition of 25 ml of methanol/water (70/30); sample size may be increased if required, but the volume of water must be adapted accordingly: e.g. 10 g in 50 ml of methanol/water (70/30)
Shaking	Blending the sample by ultra-turrax (or equivalent for 2 minutes or shaking vigorously for three minutes (manually or with shaker)	Shaking vigorously for three minutes (manually or with shaker)
Filtering / centrifuging	Filtering the extract through Whatman No. 1 filter (or equivalent)	Centrifuging the extract: 10 min / 3500 g / room temperature (20 – 25 ºC) or filtering the extract through filter (Whatman No.1 filter)
Diluting	Diluting the filter sample extract 1:4 (1+3) with distilled water (e.g. 1 ml of the extract + 3 ml of distilled water)	Diluting the supernatant or filtrate 1:7 (1+6) with sample dilution buffer (buffer 1)
Filtrate / supernatant	Using 50 µl of the filtrate per well in the test	Using 50 µl of diluted supernatant or filtrate per well in the test

Test implementation includes procedures followed:

1. Insertion of a sufficient number of wells into the microwell holder for all standards and sample to be run. Recording standard and sample positions.

2. Pipetting of 50 µl of standard (solutions) or prepared sample into separate wells; using a new pipette tip for each standard or sample.

3. For determining DON: addition of 50 µl of enzyme conjugate (red cap) to each well.

4. Addition of 50 µl of anti-DON antibody solution (black cap) to each well (50 µl of the diluted enzyme to each well for ZEA). Mixing gently by shaking the plate manually and incubating for 5 min (+/- 1) at room temperature (20–25 ºC); the specific reaction starts with the addition of the specific antibody.

5. Pouring the liquid out of the wells into a sink. Taping the microwell holder upside down vigorously against absorbent paper/onto a clean filter towel (three times in a row) to ensure complete removal of liquid from the wells. Using a wash bottle or multichannel pipette, fill the wells (250 µl per well) with washing buffer-buffer salt from test kit (with distilled water for ZEA). Empty the wells again and remove all remaining liquid. The washing procedure must be repeated two more times.

6. Addition of 100 µl of substrate/chromogen (brown cap) to each well. Mixing gently by shaking the plate manually and incubate for 3 minutes (+/- 0.5) for DON (for 30 minutes for ZEA) at room temperature (20 – 25 ºC) in the dark.

Time requirement for test implementation (incubation time) for 10 samples is 8 minutes for determining DON and 150 minutes for ZEA.

A specific software, the RIDA®SOFT Win (Art. No. Z9999), is available for evaluation the RIDASCREEN® enzyme immunoassays (for single determinations logit/log evaluation and for double or multiple determinations cubic spline should be used). The course of the standard curve is shown in the Quality Assurance Certificate enclosed in the test kit.

For the calculation without software the percentage of absorbance is calculated according to the equation:

Percent (%) absorbance = (absorbance standard (or sample) / absorbance zero standard) x 100

The zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages. The values calculated for the standards are entered in a system of coordinates on semi-logarithmic graph paper against the DON concentration ($mgkg^{-1}$) ZEA concentration (μgkg^{-1}).

The DON concentration in $mgkg^{-1}$ corresponding to the extinction of each sample can be read from the calibration curve.

The ZEA concentration in μgkg^{-1} actually contained in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor. When working in accordance with the regulation stated, the dilution factors is 35.

Since in the first year (2006) observation (2006) no contamination of grains either with DON (limit of detection = 200 µg DON kg^{-1} flour) or with ZEA (limit of detection = 5 µg ZEA kg^{-1} flour) was detected, the statistical evaluation of results was performed only for the second year (2008). The data on the contamination of white and dark flour in dependence of production method were then analysed by the method of statistic variance. First the transformation value (*tv*) was made for all data using the formula: *tv = sqr(x+½)* so that we were able to evaluate the data statistically using the analysis of variance for the normal distribution of data (Weber, 1961).

3 RESULTS

3.1 Mycotoxin DON found on the wheat grain samples of the cultivars ‘Reska’ and ‘Savinja’

On the location of IOSDV Jable in 2006 the mycotoxin DON was not detected in any of both cultivars (in frame of detection). White and dark bread baked from the flour of both cultivars would be completely safe according to the EU criterion concerning the safety of flour from contamination with DON (European Commission, 2006) irrespective of any production method included in the experiment.

Contamination of flour with the mycotoxin DON was detected in both cultivars in the intensive methods of production under weather conditions in 2008 when an above the average precipitation quantity occurred on the IOSDV Jable location (1593 mm in 2008, 1064 mm in 2006; SURS (Statistical Office of the Republic of Slovenia), 2011). In the cultivar ‘Reska’ grown in the method with stable manure and N-min (BN2) the white flour was contaminated in two out of three repetitions. In the same flour type the CN2 production method was contaminated in all three repetitions (see Table 2) while the dark flour of the same cultivar was contaminated with DON in all three repetitions of intensive production (BN2, CN2). Within the limits of possible determination using the method chosen in the cultivar ‘Savinja’ the white flour was contaminated with DON in all three repetitions of the method BN2 and two repetitions in dark flour (Table 2). The methods of production without fertilization with N-min (AN0, BN0, CN0) were not contaminated with DON in any of flour types belonging to both wheat cultivars.

Table 4: White and dark flour* contamination with the mycotoxin DON above the detection level (200 µg DON kg-1 flour) in the cultivars 'Reska' and 'Savinja' on IOSDV Jable in 2008 (µg DON kg-1 flour)

Preglednica 4: Kontaminacija bele in črne moke* z miktoksinom DON nad mejo detekcije (200 µg DON kg^{-1} moke) pri kultivarjih 'Reska' in 'Savinja' na IOSDV Jable v 2008 (µg DON kg^{-1} flour)

µg DON kg^{-1} flour	'Reska'								'Savinja'							
	White flour*				Dark flour*				White flour				Dark flour			
Block	I	II	II	Mean	I	II	III	Mean	I	II	I	Mean	I	II	III	Mea
AN0	-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BN0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CN0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BN2	250	-	630	293	630	260	1000	630	330	270	530	477	220	-	500	240
CN2	570	300	670	513	670	280	250	400	-	-	-		-	-	-	-

*White flour of the type 405, dark flour of the type 1050
** <200 µg DON kg^{-1} flour

For practical and theoretical reasons it is important to know whether the occurrence of mycotoxin DON in wheat flour is influenced statistically significantly by the flour type, the method of production and the cultivar. The relevant analysis is presented in the Table 5. From the presentation it is evident that the contamination with the mycotoxin DON (at $p \leq 0.05$) of both the white and the dark flour of the cultivar 'Reska' is significantly influenced by the method of production.

Table 5: Significance of contamination of wheat grain (white and dark flour*) of two cultivars with the mycotoxin DON in dependence on production methods, cultivar ('Reska' and 'Savinja') and flour type; IOSDV Jable, 2008; transformed values of contamination $\sqrt{(1/2 + x)}$

Preglednica 5: Značilnost kontaminacije pšeničnega zrnja z mikotoksinom DON v odvisnosti od metode pridelovanja, kultivarja ('Reska' in 'Savinja') in tipa moke, IOSDV Jable, 2008; transformirane vrednosti $\sqrt{(1/2 + x)}$

Variability source	'Reska'				'Savinja'			
	White flour		Dark flour		White flour		Dark flour	
	F-test	Significance	F-test	Significance	F-test	Significance	F-test	Significance
Blocks	1.03	$p \geq 0.05$	1.00	$p \geq 0.05$	1.00	$p \geq 0.05$	0.28	$p \geq 0.05$
Methods	6.88	$p \leq 0.05$	22.26	$p \leq 0.00$	88.22	$p \leq 0.05$	3.52	$p \geq 0.05$

*White flour of type 405, dark flour of type 1050

In cultivar 'Savinja' the contamination of white flour with mycotoxin DON depends significantly on the production methods while in dark flour the differences are not statistically significant (Table 5).

From the results presented in Tables 4 and 6 it is evident that in extensive production methods with no N-min fertilization neither white nor dark flour of any of the two cultivars included in the experiment were contaminated with the mycotoxin DON. Since in the dark flour of the cultivar 'Savinja' even in intensive production methods (BN2, CN2) the content of DON did not increase statistically significantly in comparison to the extensive production methods (AN0, BN0, CN0) and in the white flour of this cultivar only that in the production method BN2 was contaminated

with DON statistically significantly, we have concluded that the cultivar 'Savinja' was more resistant to the contamination of grains with DON than the cultivar 'Reska'. In the latter, both intensive production methods (BN2, CN2) were contaminated with DON more significantly than the extensive methods (AN0, BN0, CN0) (Table 6).

Table 6: Statistical difference between the contamination with DON in various production methods in white and dark flour* of the cultivars 'Reska' and 'Savinja', IOSDV Jable, 2008; transformed values of contamination $\sqrt{(1/2 + x)}$

Preglednica 6: Statistična razlika med kontaminacijo z DON pri različnih metodah pridelovanja v beli in črni moki kultivarjev 'Reska' in 'Savinja', IOSDV Jable, 2008; transformirane vrednosti kontaminacije $\sqrt{(1/2 + x)}$

Transformed values of mycotoxin DON (√1/2+x)				
Production methods	'Reska'		'Savinja'	
	White flour	Dark flour	White flour	Dark flour
BN2	14.14 a**	24.29 *a*	18.87 a	13.10 a
CN2	19.32 a	19.49 *a*	0.71 b	0.71 a
CN0	0.71 b	0.71 *b*	0.71 b	0.71 a
BN0	0.71 b	0.71 *b*	0.71 b	0.71 a
AN0	0.71 b	0.71 *b*	0.71 b	0.71 a

*White flour of type 405, dark flour of type 1050

* Values designated with the same letter do not differ statistically significantly at $p = 0.05$ (Duncan's test);

Since the white flour of the cultivar 'Savinja' was evidentiary contaminated with DON in all three repetitions of the method BN2 and the dark flour only in two of them (Table 4) it is logical to speculate whether the difference between the contamination of dark and white flour was statistically significant. Testing of the characteristics of differences between the contamination of dark and white flour in the method BN2 was performed using the 'method of pairwise comparison' (Weber, 1961). The test showed that the contamination of white and dark flour with DON in this production method did not differ statistically at $p \leq 0.05$ (Table 7).

Table 7: Testing differences in the contamination with the mycotoxin DON between three repetitions of samples of white (w) and three repetitions of samples of dark (d) flour of the cultivar 'Savinja' in IOSDV Jable in 2008; transformed value of infection $\sqrt{(½ + x)}$

Preglednica 7: Razlike v kontaminaciji z mikotoksinom DON med vzorci treh ponovitev bele (w) in treh ponovitev črne (d) moke kultivarjev 'Savinja' v IOSDV Jable 2008; transformirane vrednosti kontaminacije $\sqrt{(½ + x)}$

Method	Arithmetic mean	Variance	(x_1-x_2)	$T_{izr(0,05)}$	$T_{tab(0,05)}$	Significance (p=0.05)
wBN2*	19,22	11,65	6,58	1,93	2,78	No
dBN2**	12,64	120,99				

*White flour, method BN2

**Dark flour, method BN2

3.2 Mycotoxin ZEA found on the wheat grain samples of the cultivars 'Reska' and 'Savinja'

The contamination of wheat flour with mycotoxin ZEA was not detected in any of the production methods on the location of IODSV Jable in 2006. However, in the year 2008 mycotoxin ZEA appeared in the dark flour samples of the cultivar 'Reska' (within the limit of detection <5 µg ZEA kg^{-1} flour) in all repetitions of intensive production methods (BN2, CN2) and in one repetition of extensive method in which the stable manure without min-N fertilization had been applied (BN0). From the Table 10 it is evident that in the white flour of the cultivar 'Reska' the mycotoxin ZEA did not occur in any of the production methods (in frame of the limit of detection using the method chosen). The dark flour of the cultivar 'Reska' was contaminated with ZEA in all three repetitions of both intensive production methods (BN2, CN2) and only one repetition of the method BN0 among extensive production methods. Based on the above findings it may be concluded that in both intensive production methods of the cultivar 'Reska' contamination with the mycotoxin ZEA was significantly more expressed in the dark flour than in the white one in which within the limit of detection using the chosen method the mycotoxin ZEA was not detected.

In the cultivar 'Savinja' the contamination with ZEA was proven in lesser number of samples than in the cultivar 'Reska' and it was less dependent on the production methods (Table 8). The differences between the occurrence of mycotoxin ZEA in dependence on production method and flour type in the cultivar 'Savinja' were not as pronounced as in the cultivar 'Reska' and the occurrence of the mycotoxin ZEA was less frequent, too. The statistical significance of the influence of production methods on the contamination of flour with the mycotoxin ZEA is presented in Table 9.

Table 8: Contamination of white and dark flour* (<5 µg ZEA kg^{-1} flour) of the cultivars 'Reska' and 'Savinja' with the mycotoxin ZEA in dependence on the production methods in IOSDV Jable, year 2008 (µg ZEA kg^{-1} flour)

Preglednica 8: Kontaminacija bele in črna moke* (<5 µg ZEA kg^{-1} moke) kultivarjev 'Reska' in 'Savinja' z mikotoksinom ZEA v odvisnosti od načina kultivacije, IOSDV Jable, 2008 (µg ZEA kg^{-1} moke)

µg ZEA kg^{-1} flour	'Reska'								'Savinja'							
	White flour				Dark flour				White flour				Dark flour			
Block	I	II	III.	Mean	I	II	III	Mean	I	II	III.	Mean	I	II	III	Mean
AN0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BN0	-	-	-	-	14	-	-	4.7	-	-	-	-	-	-	-	-
CN0	-	-	-	-	-	-	-	-	-	-	-	-	22	-	-	7.3
BN2	-	-	-	-	16	9	79	38	-	7	-	2.3	18	44	-	20.7
CN2	-	-	-	-	22	3	12	15.7	-	-	-	-	6	-	-	2.0

<5 µg ZEA kg^{-1} flour
*White flour of the type 405, dark flour of the type 1050

Table 9 displays a statistically significant influence of production methods in the dark flour of the cultivar 'Reska' on the occurrence of the mycotoxin ZEA. In the cultivar 'Savinja', too, ZEA occurred more often in the dark flour than in the white one. Using F-test did not help prove a significant influence of production methods on the occurrence of mycotoxin ZEA either in dark flour or in the white one.

Table 9: Significance of contamination of white and dark flour of two cultivars with the mycotoxin ZEA in dependence on production methods; IOSDV Jable, 2008; transformed values of contamination $\sqrt{(1/2 + x)}$

Preglednica 9: Značilnost kontaminacije bele in črne moke dveh kultivarjev (‘Reska’ in ‘Savinja’) z mikotoksinom ZEA v odvisnosti od metode pridelovanja; IOSDV Jable, 2008; transformirane vrednosti $\sqrt{(1/2 + x)}$

Variability source	‘Reska’ Dark flour		‘Savinja’ White flour		‘Savinja’ Dark flour	
	F-test	Significance	F-test	Significance	F-test	Significance
Blocks	0.46	$p \geq 0.05$	0.94	$p \geq 0.05$	1,67	$p \geq 0.05$
Methods	6.12	$p \leq 0.00$	1.75	$p \geq 0.05$	1,92	$p \geq 0.05$

Table 10: Significance of statistical differences between production methods as to the contamination of white and dark flour* with the mycotoxin ZEA in two wheat cultivars; IOSDV Jable, 2008; transformed values of contamination $\sqrt{(1/2 + x)}$

Preglednica 10: Značilnost statističnih razlik med postopki pridelovanja glede na kontaminacijo bele in črne moke z mikotoksinom ZEA pri dveh kultivarjih pšenice; IOSDV Jable, 2008; transformirane vrednosti $\sqrt{(1/2 + x)}$

Production methods	‘Reska’ White flour	Dark flour	‘Savinja’ White flour	Dark flour
BN2	0.71	5.80 *a**	1.38 a	3.89 a
CN2	0.71	3.98 *a*	0.71 a	1.31 a
CN0	0.71	0.71 *b*	0.71 a	2.05 a
BN0	0.71	1.74 *b*	0.71 a	0.71 a
AN0	0.71	0.71 *b*	0.71 a	0.71 a

Values designated with the same letter do not differ statistically significantly at $p = 0.05$ (Duncan's test);
*White flour of type 405, dark flour of type 1050

Results presented in Table 10 show a significantly higher degree of contamination of the dark flour of the cultivar ‘Reska’ with the mycotoxin ZEA in the two intensive production methods (BN2, CN2) than in the extensive methods (AN0, BN0 and CN0) while the white flour was not contaminated with ZEA in any of the methods.

The cultivar ‘Savinja’ also shows higher degree of contamination found in the two intensive methods than in the extensive ones (with the exception of ZEA found in the dark flour of the method CN0); however, it was not possible to prove the significance of the differences using the F-test. The use of statistical method of pairwise comparison (t-test) has shown that in the method BN2 in which the wheat flour had the highest degree of contamination with mycotoxin ZEA, this contamination was significantly higher in the dark flour than in the white one (Table 11).

Table 11: The difference in the contamination with mycotoxin ZEA between white (w) and dark (d) flour variants BN2 of the cultivar 'Savinja' on the site IOSDV Jable in 2008 (t-test); transformed values of contamination $\sqrt{(1/2 + x)}$

Preglednica 11: Razlika v kontaminiranosti z mikotoksinom ZEA med belo moko (w) postopka BN2 in moko (d) postopka BN2 pri kultivarju 'Savinja' na lokaciji IOSDV Jable leta 2008 (t-test); transformirane vrednosti $\sqrt{(1/2 + x)}$

Method	1st block	2nd block	3rd block	Arith. mean	Variance	$(x_1 - x_2)$	Tcomp	Ttab	Sign.
wBN2	0.71	2.74	0.71	1.39	3.10	3.89	2.56	2.45	Yes*
dBN2	4.30	6.67	0.71	3.89	32.16				

*At $p \leq 0.05$

4 DISCUSSION

The results obtained in the investigation have shown the contamination of wheat flour to be dependent on all the factors studied: from the influence of production methods, cultivar and year of production to the weather. The year of production had a great influence on the occurrence of the mycotoxins DON and ZEA. Since the experiment was conducted on the same location in both years with the same two cultivars and equal production methods (with regard to the degree of intensity), the fact that DON and ZEA occurred only in 2008 and not in 2006 proves that the weather had a significant impact on the occurrence of both mycotoxins. This finding is in accordance with the investigations in which the impact of weather on the contamination of wheat grain with the mycotoxins DON and ZEA was established (Whitlow and Hagler, 2009, Prandini et al., 2009). Some other researchers talk about the impact of location (Zemljič et al., 2008), but also their findings can be related to the influence of weather or climate as other location may also imply other weather conditions.

As it is almost impossible to influence the yearly weather conditions, from the producer's standpoint the result saying that the production method has also a great influence on the occurrence of DON and ZEA is more useful. We have proved that in this frame an important role is played by the intensity of N-min (mineral nitrogen) fertilization. In both cultivars, the methods with medium rate of N-min (130 kg N ha^{-1}) were as a rule significantly more contaminated than the production methods with no N-min fertilization. In an investigation conducted in similar natural conditions (Zemljič et al., 2008) it was impossible to establish whether fertilization with N-min had an impact on the occurrence of mycotoxin DON, the content of which was studied in wheat grains. However, opposite to our investigation conducted under the circumstances of a permanent field experiment, their field experiment was conducted only one year and only in variants of more or less intensive methods and not in the variants of extensive ones. Since without nitrogen fertilization the mineralization of organic matter in the soil decreases to the point in which the fertilization with nitrogen becomes more pronounced, static permanent field experiments with diversified nitrogen rates which also include variant with no nitrogen fertilization allow better options (Smith et al., 2001).

For a safe production and use of food the results obtained with the grinding of wheat grain in white (type 400) and dark flour (type 1000) are important as well as the establishment of difference between them as far as the contamination with DON and ZEA are concerned. With the extensive production methods no statistically significant differences were found between the white and dark flour in the sense of contamination with DON, however, in this respect there was a significant difference in the

intensive production methods. With the cultivar 'Reska' the white flour was not proven to be ZEA contaminated in all production methods but with the cultivar 'Savinja' the dark flour in the method BN2 (the only method in which the mycotoxin ZEA occurred in the white flour) was significantly more contaminated with the mycotoxin ZEA than the white flour. As for babies the safety threshold lies at 20 µg ZEA kg^{-1} flour, so the dark flour grown in the method of wheat fertilization with 130 kg N ha^{-1} was not safe enough for baby food in view of the fact that the average content of mycotoxin ZEA in dark flour of the method BN2 in both cultivars exceeded the allowable 20 µg ZEA kg^{-1} flour. If we want to consume healthy bread, considering the results of the present study we should avoid eating dark bread which the public considers to be healthier than the white one.

The content of mycotoxin DON in white wheat flour of the cultivar 'Reska' did not exceed in any sample the allowable content of 750 µg DON kg^{-1} flour which is the limit value valid for adult persons. However, the content of 200 DON kg^{-1} of flour, which is the limit value for safe food for children, was exceeded in white flour in all three repetitions of the method CN2 and in two repetitions of the method BN2. In the dark flour of the cultivar 'Reska' in one repetition of the method BN2 the allowable limit value for food safety for adults, which is 750 DON kg^{-1} flour, was exceeded by 250 DON kg^{-1} flour. In the remaining repetitions of intensive production methods (BN2, CN2) the DON content exceeded the limit value for food safety for children while in extensive production methods both the mycotoxin DON content was not detected either in white or in dark flour samples.

In the flour of the cultivar 'Savinja' the content of DON mycotoxin were detected only in the BN2 method, i.e. in three repetitions they were higher in white flour than the limit value of safe food for children. In dark flour only two repetitions of the method BN2 were contaminated with the mycotoxin DON, their content being above the limit of food safety for babies. All extensive production methods did not present any detectable contamination with the mycotoxin DON. The presumption that there was less mycotoxin DON in white flour than in the dark one was not confirmed, however, an increasing tendency towards Savinja as being less susceptible to the contamination with this mycotoxin than Reska has been observed.

Beside fertilization with N-min the contamination with DON and ZEA was also significantly influenced by the fertilization with organic fertilizers. The comparison of the methods BN2 and CN2 has shown that the contamination with DON was as a rule higher in BN2 than in CN2. One of the main reasons was the exceeded total N (nitrogen) in the method BN2 in comparison with CN2 as the ploughed straw contains less N (nitrogen) than the ploughed stable manure.

The comparison of the resistance of both cultivars to contamination with DON and ZEA has shown that the cultivar 'Savinja' was more resistant to the contamination with the two mycotoxins studied than the cultivar 'Reska'. The cultivar 'Reska' witnessed thus in the method including fertilization with FM and N-min (BN2) on the average of the repetitions an almost three times (2.6 times) higher contamination with DON than the cultivar 'Savinja' while in the method with straw (CN2) a contamination with DON in this cultivar was not observed at all. Similar to that, the contamination with ZEA in the cultivar 'Reska' was almost twice (1.84 times) as high in the method BN2 and almost eight times higher in the method CN2 than in the cultivar 'Savinja'.

5 CONCLUSIONS

Based on the findings of the current investigation it may be concluded that adequately chosen agro-technical measures significantly reduce the risk of excessive contamination of foodstuffs of cereal origin with the mycotoxins DON and ZEA. In this frame a too abundant fertilization with N (nitrogen) of either inorganic or organic origin is to be avoided. The extensive wheat production implies an appreciably lesser risk of DON and ZEA occurrence than the intensive one. With regard to the results obtained it may be assumed that the occurrence of the mycotoxin ZEA is less dependent on the intensity of N (nitrogen)

fertilization than the occurrence of the mycotoxin DON.

The influence of various production methods on the contamination of wheat grain with the mycotoxins DON and ZEA may be reliably confirmed only in frame of a long-term field experiment which includes both intensive and extensive methods of N (nitrogen) fertilization.

In spite of the fact that two cultivars were included in the current investigation we were able to prove that the cultivars differ with regard to resistance to the contamination with the mycotoxins DON and ZEA. It would therefore require establishing the susceptibility of individual cultivars to the contamination with mycotoxins in order to be able to avoid those more susceptible to it.

The conclusion saying that dark flour which is usually recommended as healthier was more contaminated with the mycotoxins DON and ZEA than the white one would also require further examination. As far as the presence of bran in various foods it would be worth while studying the content of both mycotoxins in bran.

6 REFERENCES

Atroshi F., Rizzo A., Westermack T., Ali-Vehmas T. 2002. Antioxidant nutrients and mycotoxins. Journal of Toxicology, 180: 151–167. DOI: 10.1016/S0300-483X(02)00388-8

Casteel S.W., Rottinghaus G.E. 2000. Mycotoxicoses. V: Encyclopedia of microbiology. Vol. 3. 2nd ed. Lenderberg J. (ed). San Diego, Academic Press: 337–348

Commission Regulation (EC) no. 466/2001. Working document. 2004. Brussels, Sanco, 6: 7-8 https://www.fsai.ie/uploadedFiles/Legislation/Consultations/120804_eu_proposal.pdf

Commission Regulation (EC) no. 401/2006a of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. 2006. Official Journal of the European Union, 49, L 70: 12-34

Commission Regulation (EC) no. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. 2006. Official Journal of the European Union, 49, L 364: 5-24

Commission Regulation (EC) no. 1126/2007 of 28 September 2007 amending Regulation (EC) no. 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. 2007. Official Journal of the European Union, 50, L 255: 14-17

Čergan Z., Tajnšek A. 2010. Dolgoročni učinek (1993–2009) diferenciranega gnojenja z organskimi gnojili in mineralnim dušikom na pridelek in gospodarnost treh poljščin v kolobarju na dveh lokacijah. V: Novi izzivi v poljedelstvu 2010. Zbornik simpozija. Rogaška Slatina, 2010. Kocjan Ačko D., Čeh B. (ur.). Ljubljana, Slovensko agronomsko društvo: 42–48

European Commission. 1999. Opinion on the relationship between the use of plant protection products on food plants and the occurrence of mycotoxins in foods. SCP/RESI/063. Brussels, European Commission, Health & Consumer Protection Directorate-general, Scientific Committee on Plants: 24 p. http://ec.europa.eu/food/fs/sc/scp/out56_en.pdf

Forrer H.R., Musa T., Schwab F., Jenny E., Bucheli T.D., Wettstein F.E., Vogelgsang S. 2014. Fusarium Head Blight Control and Prevention of Mycotoxin Contamination in Wheat with Botanicals and Tannic Acid. Toxins, 6, 3: 830-849. DOI: 10.3390/toxins6030830

Gregorčič A., Velikonja Bolta Š., Verbič J., Kmecl V., Žnidaršič Pongrac V., Sušin J. 2009. Analiza prisotnosti onesnaževal v živilih rastlinskega izvora v primarni proizvodnji. Naročnik Ministrstvo za kmetijstvo, gozdarstvo in prehrano. Ljubljana, Kmetijski inštitut Slovenije: 34 p.

Grižon P., Per M., Pečnik M. 2011. Sortna lista poljščin, zelenjadnic, sadnih rastlin in trte za leto 2011. Ljubljana, RS MKGP, Fitosanitarna uprava RS: 26 p. http://www.r-biopharm.com/de/.

Hueza I.M., Raspantini P.C.F., Raspantini L.E.R., Latorre A.O., Gornjak S.L. 2014. Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. Toxins, 6, 3: 1080–1095; http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3968378/ (6. jul. 2014). DOI: 10.3390/toxins6031080

In vitro Test. R-Biopharm AG, Darmstadt, Germany (document provided by LUFA Speyer)

Jakovac-Strajn B., Kotnik V., Vengušt A., Pestevšek U., Malovrh T. 2004. Učinek mikotoksina

deoksinivalenola na imunski odziv prašičev. Medicinski razgledi, 43, Suppl.5: 167–171

Jakovac-Strajn B., Vengušt A., Ujčič Vrhovnik I., Pavšič Vrtač K., Kalcher-Tavčar G. 2010. Mikrobiološke in mikotoksikološke preiskave žitaric v Sloveniji. Acta agriculturae Slovenica, 95, 2: 121– 28

Kalcher-Tavčar G., Pavšič Vrtač K., Pestevšek U., Jakovac-Strajn B., Ujčič I., Vengušt A. 2007. Kontaminacija krme z nekaterimi mikotoksini v Sloveniji. V: Zbornik predavanj, 16. mednarodno znanstveno posvetovanje o prehrani domačih živali, Radenci, 8.- 9.11.2007. Kapun S., Krambereger B., Čeh T. (eds). Murska Sobota, Kmetijsko gozdarska zbornica Slovenije, Kmetijsko gozdarski zavod: 52–60

Nogueira da Costa A., Mijal R.S., Keen J.N., Findlay J.B., Wild C.P. 2011. Proteomic analysis of the effects of the immunomodulatory mycotoxin deoxynivalenol. Proteomics, 11, 10: 1903-1914; http://www.ncbi.nlm.nih.gov/pubmed/21462343?dopt=Abstract. DOI: 10.1002/pmic.201000580

Pavlič Nikolič E. 2005. Vpliv gnojenja pšenice (Triticum aestivum L.) cv. 'Reska' v različnih ekoloških območjih na kakovost zrnja. Magistrsko delo. Ljubljana, Biotehniška fakulteta, Oddelek za agronomijo: 81 p.

Prandini A, Sigolo S, Filippi L, Battilani P, Piva G. 2009. Review of predictive models for *Fusarium* head blight and related mycotoxin contamination in wheat. Food Chem Toxicol 47:927-931. DOI: 10.1016/j.fct.2008.06.010

Ristić, Z., Bukovnik, U., Vana Prasad P. U. 2007. Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under heat stress. Crop Science, 47:2067-2073. DOI: 10.2135/cropsci2006.10.0674

Schachermayr G., Fried P. M. 2000. Problemkreis Fusarien und ihre Mykotoxine. Agrarforschung, 7: 252–257

Smith J. E., Lewis C. W., Anderson J. G., Solomons G. L. 1994. Mycotoxins in human nutrition and health. Brussels, European Commission Directorate - General XII: 300 p

Smith P., Falloon P., Smith J. U., Powlson D. S. 2001. GC&TE Global change and terrestrial ecosystems. Report 7, Second Edition, Soil Organic Matter Network (SOMNET): 2001 Model and Experimental Metadata, Walingford, UK, 2001: 221 p

Srey C., Kimanya M.E., Routledge M.N., Shirima C.P., Gong Y.Y. 2014. Deoxynivalenol, exposure assessment in young children in Tanzania. Molecular Nutrition and Food Research, 58, 7: 1574-1580. DOI: 10.1002/mnfr.201400012

SURS. 2011. Povprečne letne in mesečne temperature zraka po meteoroloških postajah Slovenije. Ljubljana, Statistični urad RS: 1str. http://pxweb.stat.si/pxweb/Dialog/varval.asp?ma=0156101S&ti=&path=../Database/Okolje/01_ozemlje_podnebje/10_01561_podnebni_kazalniki/&lang=2 (november 2011)

Tajnšek A. 2003. Namen in cilj trajnih poljskih poskusov IOSDV Jable in OISDV Rakičan. V: Deset let trajnih poskusov IOSDV v Sloveniji, Jable in Rakičan 1993-2003: zbornik posveta, Žalec, december 2004. Tajnšek A., Čeh Brežnik B., Kocjan Ačko, D. (eds). Ljubljana: Slovensko agronomsko društvo: 7-22. DOI: 10.1080/03650340902737888

Tajnšek L. 2004. Vpliv gnojenja pšenice cv. Soissons in cv. Eko v kolobarju na nekatere pekarske lastnosti pšenice. Diplomsko delo. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo: 43 p.

Tajnšek L., Tajnšek A. 2004. Vpliv gnojenja pšenice na nekatere pekarske lastnosti pšenice in odkupno ceno pri invertnem odkupu. V: Novi izzivi v poljedelstvu 2004. Zbornik simpozija. Čatež ob Savi, 13. in 14. december 2004. Tajnšek A. (ed). Ljubljana, Slovensko agronomsko društvo: 240–245

Tajnšek A., Nikolič Pavlič E., Čeh B., Tajnšek L. 2010. Baking quality of wheat grain with regard to agrotechnical arrangements and location. Archives of Agronomy and Soil Science, 56, 5: 589–603

Tajnšek A., Tajnšek L. 2011. Gorolka in Savinja, dve novi slovenski sorti pšenice. Hmeljarski bilten, 18: 65–71

Tajnšek A., Čergan Z., Čeh B. 2013. Results of the long-term field experiment IOSDV Jable at the beginning of 21 st century.) Archives of Agronomy and Soil Science, 59, 8: 1099–1108. DOI: 10.1080/03650340.2012.697996

Usleber E., Maertlbauer E., Dietrich R., Terplan G. 1991. Direct enzyme - linked immunosorbent assays for the detection of the 8 – ketotrichothecene mycotoxins deoxinivalenol, 3 – acetyldeoxynivalenol, and 15 – acetyldeoxynivalenol in buffer. V: Journal of agricultural and food chemistry. Washington, DC (u.a.), ISSN 1520 – 5118, Vol. 39, No.11 (1991), p. 2091 - 2095

Waśkiewicz A., Beszterda M., Kostecki M., Zielonka Ł, Goliński P., Gajęcki M. 2014. Deoxynivalenol in

the Gastrointestinal Tract of Immature Gilts under per Os Toxin Application. Toxins 2014, 6: 973-987; doi: 10.3390/toxins6030973 http://www.mdpi.com/2072-6651/6/3/973 (6. jul. 2014). DOI: 10.3390/toxins6030973

Weber E, 1961. Grundriss der biologischen Statistik. VEB Gustav Fischer Verlag, Jena, s. 235-236

Whitlow LW, Hagler JR (2009).Mycotoxins in feeds. Feedstuffs, September 16:70-78

Williams P.R., Hammitt J.K. 2001. Perceived risks of conventional and organic produce. Pesticides; pathogens and natural toxins. Risk Analytics, 21: 319–330. DOI: 10.1111/0272-4332.212114

Zemljič A. Rutar R., Žerjav M., Verbič J. 2008. Vpliv sorte, gnojenja z dušikom in razkuževanja semena na kontaminiranost zrnja pšenice s *Fusarium* spp. in onesnaženost z mikotoksini. V: Novi izzivi v poljedelstvu 2008. Rogaška Slatina, 4. in 5. december 2008. Tajnšek A. (ed). Ljubljana, Slovensko agronomsko društvo: 257–262